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SUPPLEMENTARY MATERIAL

corresponding to:

Prep1 (pKnox1) transcription factor contributes to pubertal mammary gland branching morphogenesis

LARA SICOURI, FEDERICA PISATI, SALVATORE PECE, FRANCESCO BLASI* and ELENA LONGOBARDI*

***Address correspondence to:** Elena Longobardi. Section of Pharmacology, Department of Neuroscience, University of Naples Federico II, Via S. Pansini 5, 80131 Naples, Italy. Tel: 081-7463316. Fax: 081-7463323. E-mail: elelong@libero.it - https://orcid.org/0000-0001-8283-4735
or Francesco Blasi. IFOM (FIRC Institute of Molecular Oncology), Via Adamello 16, 20139 Milan, Italy. Tel: 0039-02-574303288. Fax: 0039-02-574303222.
E-mail: francesco.blasi@ifom.eu - web: http://www.ifom.eu/en/cancer-research/research-labs/research-lab-blasi.php - https://orcid.org/0000-0001-9406-1784

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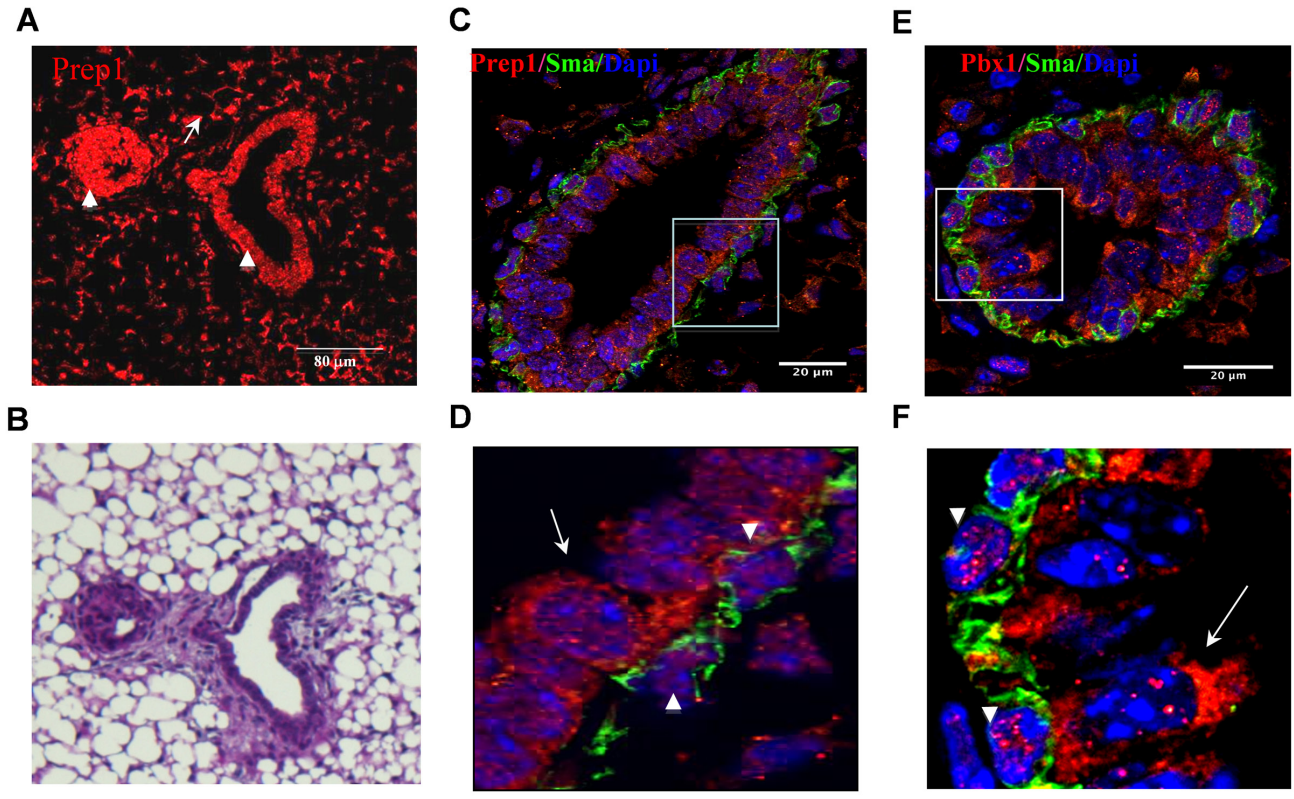
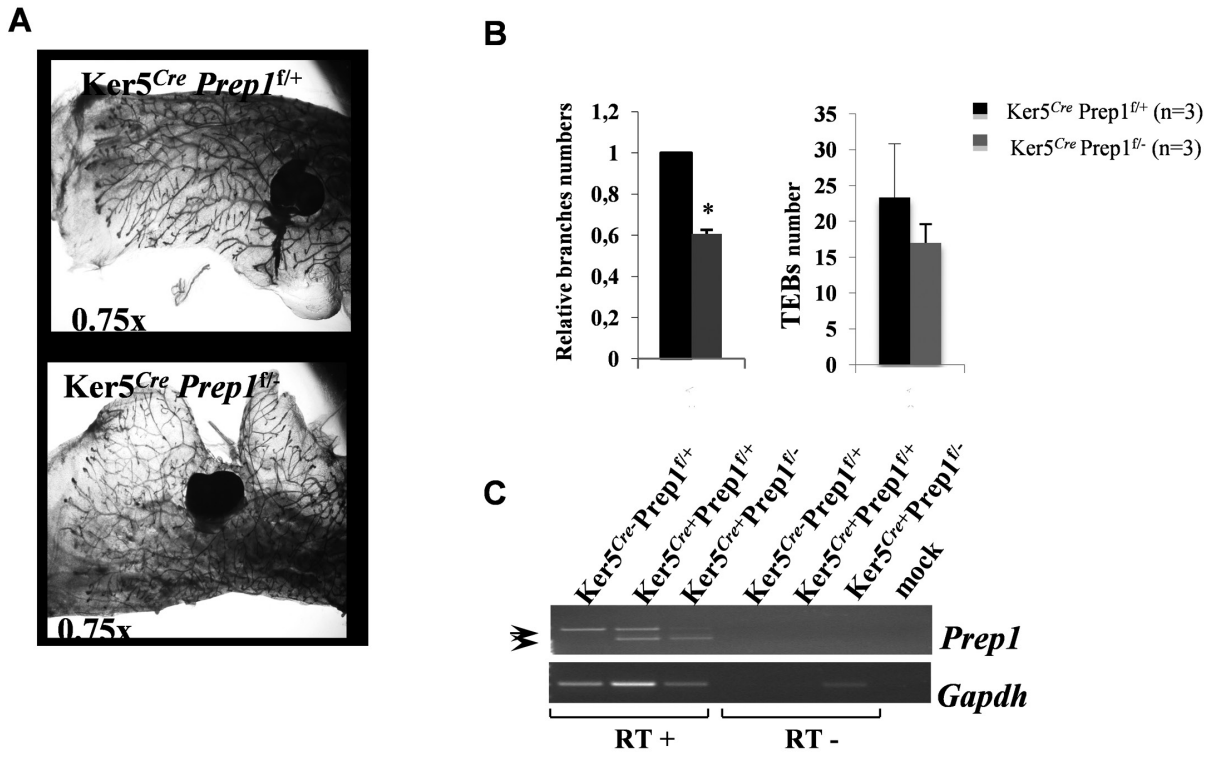
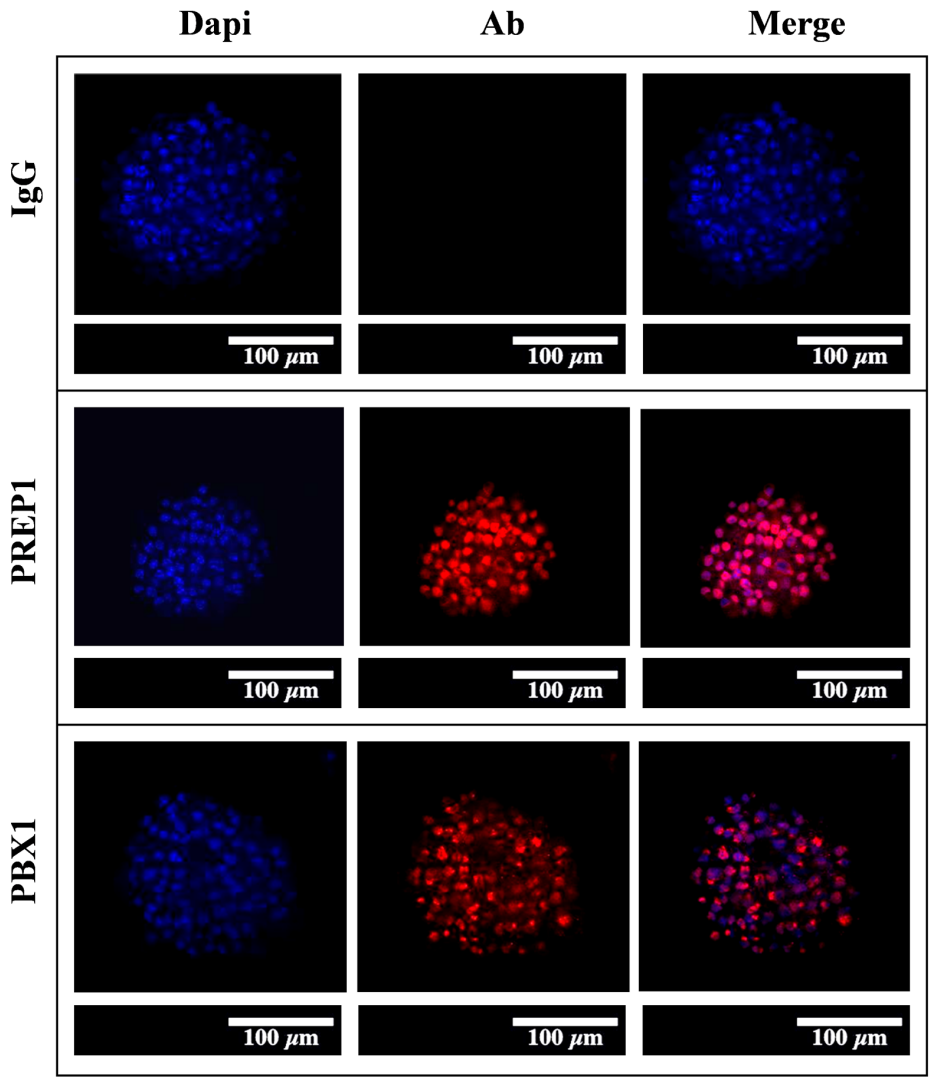


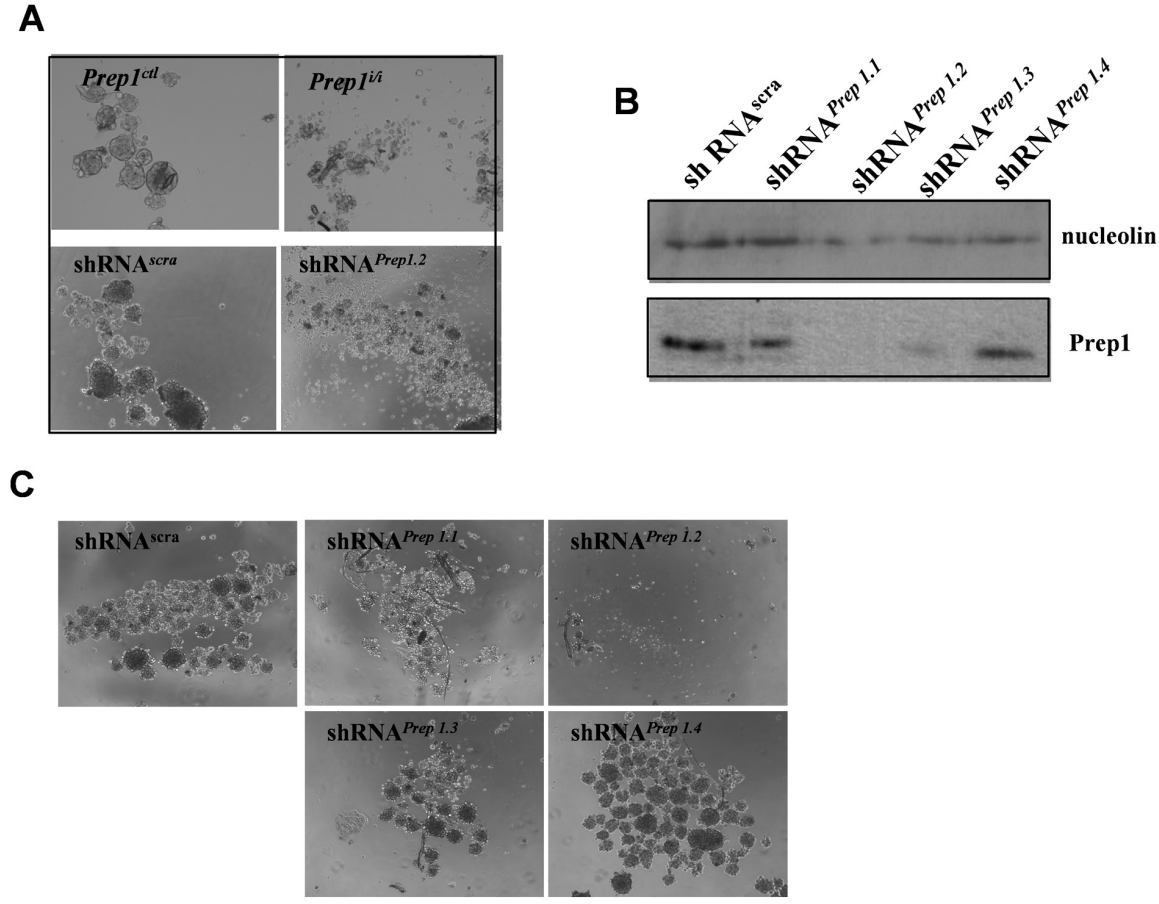
Supplementary Fig. S1. Prep1 is expressed in ducts and TEBs the mouse mammary gland. *Formalin fixed sections of mammary glands from 4-5 weeks old virgin wild-type mice were subjected to immunofluorescence with the indicated antibodies and analyzed by confocal microscopy.* **(A)** *Prep1 positive areas (red) are evident in the epithelial parenchyma (TEBs; arrowheads) and in the stroma (harrow); scale bar, 80 µm.* **(B)** *HE staining on section from A.* **(C)** *Co-immunofluorescence of Prep1 (red) and SMA (green) on mammary gland sections. Prep1 stains both luminal and SMA positive myoepithelial cells in fully differentiated ducts. Scale bars: 30 µm.* **(D)** *Higher magnification of boxed area from (C), showing a nuclear/cytoplasmic Prep1 positivity in the luminal cells (arrow) and an exclusively nuclear localization in myoepithelial cells (harrowheads).* **(E)** *Co-immunofluorescence of Pbx1 (red) and SMA (green) on mammary gland sections. Pbx1 stains both luminal and SMA positive myoepithelial cells in fully differentiated ducts. Scale bars: 30 µm* **(F)** *Higher magnification of boxed area from E, showing a nuclear/cytoplasmic Pbx1 positivity in the luminal cells (arrow) and an exclusively nuclear localization in myoepithelial cells (harrowheads).*



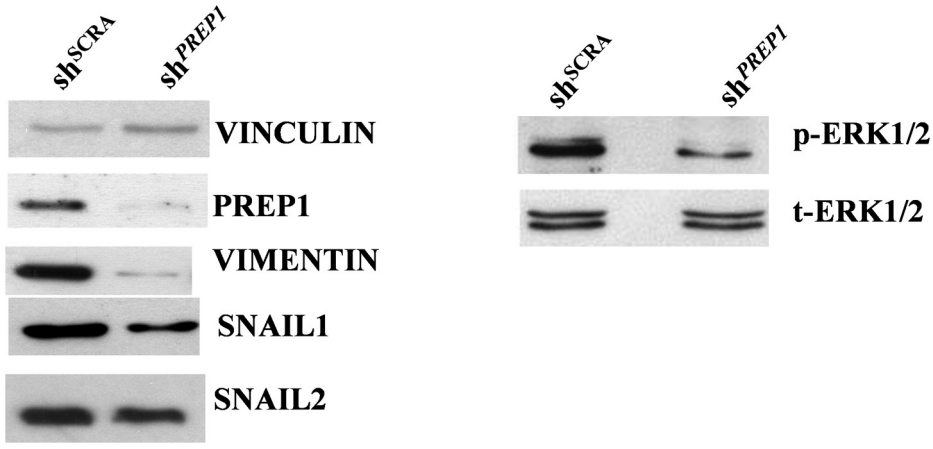
Supplementary Fig. S2. Ker5^{CrePrep1flox} conditional mice recapitulate the branching defects of the *Prep1i/i* mice. (A) *Representative Carmine Alum staining of MGs from 5 weeks-old virgin Ker5*^{CrePrep1f}*/+ and Ker5*^{Cre}Prep1$^{f/-}$ *female mice.* **(B)** *The histograms report quantifications of branches (**P<0.001*) and TEB number in Ker5*^{Cre}Prep1$^{f/-}$ *compared to Ker5*^{Cre}Prep1$^{f/+}$ *MG. In brackets the numbers of animals used for each analysis. Error bars refer to s.d. Further informations on measurements are reported in Material and Methods.* **(C)** *Residual* Prep1 *mRNA in MEC enriched fraction was evaluated by RT-PCR using Prep1-full primers; fragments corresponding to both full length (arrow) and floxed cDNA (arrowhead) were obtained according to the specific genotype. Gapdh amplicon served as loading control.*



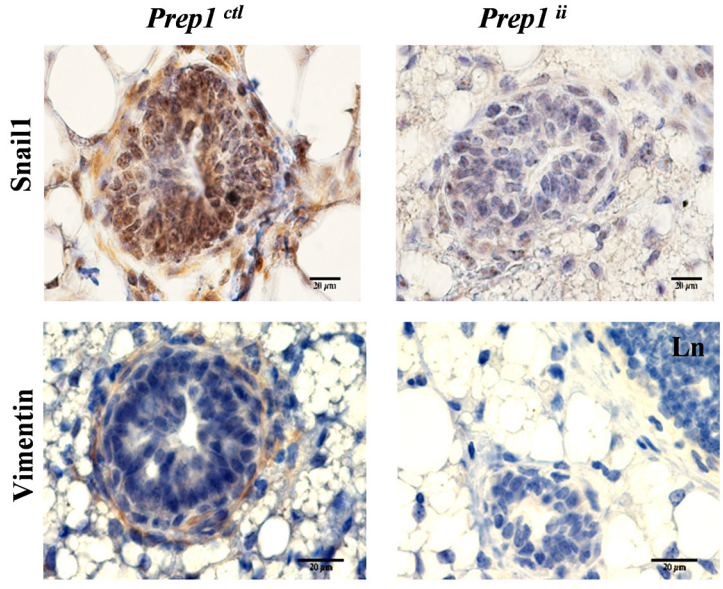
Supplementary Fig. S3. Prep1 and Pbx1 are expressed in mouse mammospheres. *Confocal acquisition of immunofluorescence with Prep1 and Pbx1 antibodies (red) on F2 mammospheres. IgG refers to sample stained only with secondary antibody. Dapi served as nuclear marker (scale bar 100 mm). Merge panels refer to Dapi merged with Prep1 or Pbx1.*



Supplementary Fig. S4. Prep1 sustains the activity of the mammary stem compartment. (A) *SFE assay: examples of passage F2 spheres obtained from MEC (mammary epithelial cells) from control and* Prep1$^{i/i}$ (top panels*) or WT mice transduced with shRNA*^{scra} *or shRNA*$^{Prep1.2}$ *lentiviruses* (bottom panels*).* **(B)** *Immunoblotting with Prep1 antibody on nuclear extracts from F2 spheres obtained upon silencing with a lentiviral vector containing scrambled shRNA (shRNA*^{Scra}*) or four different Prep1-specific shRNAs (shRNA*$^{Prep1.1}$*, shRNA*$^{Prep1.2}$*, shRNA*$^{Prep1.3}$*, shRNA*$^{Prep1.4}$*). Nucleolin was used as a loading control.* **(C)** *Representative images of the effect of Prep1 silencing on SFE: of the four tested, shRNAs*$^{Prep1.2}$ *performed best, giving the highest rate of down-regulation in immunoblots and the strongest SFE impairement in the* in-vitro *mammosphere assay.*



Supplementary Fig. S5. Prep 1 sustains ERK activity and secure physiological level of SNAIL1/2 and VIMENTIN in human MCF10A cells. *Immunostainings with PREP1, VIMENTIN, SNAILI,2, phospho ERK (p-ERK1/2) and total ERK (t-ERK1/2) antibodies on total extracts from MCF10A cells interfered with sh*^{PREP1} *or sh*^{SCRA} *viral supernatants. VINCULIN served as loading control.*



Supplementary Fig. S6. Snail1 and Vimentin are reduced in *Prep1* deficient mammary gland. *IHC on 3 mm mammary gland sections from 5 weeks-old virgin control and* Prep1$^{i/i}$ *females with Snail1 and Vimentin antibodies. Nuclei were counterstained with haematoxilin staining; Scale bars: 20 mm.*

SUPPL. TABLE S1

Antibodies	Application	Description	Source
Prep1	WB (1 μg/ml) IHC (1:50) IF (1:50)	monoclonal	Santa Cruz
Prep1	IF (1:50)	Polyclonal	Santa Cruz
SMA-FITC	IF (1:400)	Monoclonal	Sigma
Cl-Cas3	WB (1:1000)	Rabbit polyclonal	Cell Signaling
Snail2	WB (1 μg/ml) IHC (1:50)	Rabbit polyclonal	Cell Signaling
Bcl-XL	WB (1:1000)	Monoclonal	Santa Cruz
Vinculin	WB (1:20000)	monoclonal	Sigma
Actin	WB (1: 2000)	Goat polyclonal	Santa Cruz
α-goat IgG-HRP	WB (1:3500)	Rabbit polyclonal	Dako
α-Rabbit IgG-HRP	WB (1:20000)	Goat polyclonal	Biorad
α-mouse IgG-HRP	WB (1:10000)	Goat polyclonal	Biorad
Alexa Fluor® 647	IF (1:100)	Donkey α-mouse	Invitrogen
Alexa Fluor® 647	IF (1:100)	Donkey α-Rabbit	Invitrogen
Snail1	WB (1:1000)	Polyclonal	Novus
Vimentin	WB (1:1000)	Monoclonal	Santa Cruz
Erk	WB (1:1000)	Polyclonal	Cell Signaling
pErk	WB (1:1000)	Polyclonal	Cell Signaling

Primary and secondary antibodies used in this study are presented with the dilutions used for each application, a description of the type of antibody, and the companies from which these were obtained.

SUPPL. TABLE S2

LIST OF PRIMERS USED IN THIS STUDY

Primer	Sequence
Prep1	Fw: 5'-ACAGACGCTAAGTATAGACAG-3' Rw: 5'-AATCTGCTGGGATTGCACA-3'
K5	FW: 5'-AACATGCTTCATCGTCGG-3' RW: 5'-TTCGGATCATCAGCTACACC-3'
Ki67	Fw: 5'-CACCTGGTCACCATCAAGC-3' Rw: 5'-TTTGAGCCATCTGAGGCAG-3'
Cd49f	Fw: 5'-CCAGTTGTGCCTGCTCTACC-3' Rw: 5'-CTCCCATCCACTGGTCTTCC-3'
Cd24	Fw: 5'-ATGGGCAGAGCGATGGT-3' Rw: 5'-GTGGACTGCAGGGAGCTG-3'
Prep1 full	Fw: 5'-ATGATGGCGACACAGACG-3' Rw: 5'-CTACTGAAGGGAGTCGCTG-3'
Gapdh	Fw: 5'-GTCTACATGTTCCAGTATGACTCC-3' Rw: 5'-AGTGAGTTGTCATATTTCTCGTGGT-3'